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Multidrug Resistance-Associated Protein 2 (Mrp2) is Primarily Responsible for the Biliary Excretion of Fexofenadine in Mice

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Abstract

Previous studies implicated P-glycoprotein (P-gp) as the major transport protein responsible for the biliary excretion of fexofenadine (FEX). However, FEX biliary excretion was not impaired in P-gp- or Bcrp-knockout mice, and Mrp2-deficient rats. The present study tested the hypothesis that species differences exist in the transport protein primarily responsible for FEX biliary excretion between mice and rats. Livers from Mrp2-knockout (Mrp2KO) mice and Mrp2-deficient (TR⁻) rats were perfused in a single-pass manner with 0.5 μ M FEX. GF120918 (10 μ M) was employed to inhibit P-gp and Bcrp. The biliary excretion rate of FEX was decreased 85% in Mrp2KO relative to wild-type mice (18.4 ± 2.2 vs. 122 ± 34 pmol/min/g liver). In mice, more than 50% of FEX unbound intrinsic biliary clearance ($CL_{bile, int} = 3.0$ ml/hr/g liver) could be attributed to Mrp2 (Mrp2-dependent $CL_{bile, int} \sim 1.7$ ml/hr/g liver), with P-gp and Bcrp playing a minor role (P-gp- and Bcrp-dependent $CL_{bile, int} \sim 0.3$ ml/hr/g liver). Approximately one-third of FEX $CL_{bile, int}$ was attributed to unidentified mechanisms in mice. In contrast to mice, FEX biliary excretion rate (245 ± 38 and 250 ± 25 pmol/min/g liver) and $CL_{bile, int}$ (9.72 ± 2.5 and 6.49 ± 0.68 ml/hr/g liver) were comparable between Mrp2-deficient (TR⁻) and control Wistar rats, respectively, suggesting that unidentified transport mechanism(s) can completely compensate for the loss of Mrp2 function in rats. Mrp2 clearly plays a major role in FEX biliary excretion in mice. In conclusion, remarkable species differences exist in FEX hepatobiliary transport mechanisms.

Introduction

FEX is a non-sedating H₁ receptor antagonist used clinically for the treatment of seasonal allergies. FEX is metabolically stable; less than 5% of the dose is eliminated by metabolism in humans (Cvetkovic et al., 1999). FEX is eliminated primarily via biliary excretion. In mice, biliary and renal clearance account for 50–70% and 10–20% of FEX total body clearance, respectively (Tahara et al., 2005).

In vitro studies have demonstrated that cellular uptake of FEX is mediated by the 1A2 and 2B1 isoforms of human organic anion transporting polypeptide (OATP1A2 and OATP2B1, respectively); cellular efflux of FEX is mediated by human P-gp (Cvetkovic et al., 1999). FEX concentrations in plasma, brain, kidney, and liver were elevated significantly after oral and intravenous administration to P-gp-knockout mice (Cvetkovic et al., 1999). FEX has been used

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as a P-gp probe substrate for clinical drug-drug and food-drug interaction studies (Banfield et al., 2002; Shon et al., 2005).

In rodents, FEX biliary excretion was not impaired in P-gp- or Bcrp-knockout mice, or Mrp2-deficient Eisai hyperbilirubinemic rats (Tahara et al., 2005). These findings led to the hypothesis that one or more unidentified transport protein(s) distinct from P-gp, Mrp2, and Bcrp mediates the biliary excretion of FEX. Recent studies have suggested that species differences exist in transport mechanisms mediating biliary excretion (Zamek-Gliszczynski et al., 2006b). The purpose of this work was to determine whether Mrp2 is responsible for FEX biliary excretion in mice, and whether species differences in FEX biliary excretion exist between mice and rats. Furthermore, the possibility for the involvement of other transport mechanisms beyond Mrp2, P-gp and Bcrp in FEX biliary excretion was investigated with the employment of P-gp and Bcrp inhibitor GF120918 (GW918) in Mrp2-knockout mice.

Material and Methods

Chemicals

FEX, taurocholate, and Krebs-Henseleit buffer packets were purchased from Sigma Chemical Co. (St. Louis, MO). GW918 was a gift from GlaxoSmithKline (Research Triangle Park, NC). All other chemicals were of reagent grade and were readily available from commercial sources.

Animals

Male C57BL/6 (B6) wild-type and *Abcc2*($-/-$) (Mrp2KO) mice (25–30g) were provided by Eli Lilly and Co. Mrp2KO mice were generated as described previously (Nezasa et al., 2006). Male Wistar rats (250–300 g) were purchased from Charles River Laboratories, Inc. (Raleigh, NC); male Mrp2-deficient *TR*⁻ rats (obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) were bred in the UNC School of Pharmacy animal facility. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill

Single-Pass Liver Perfusion Study

In mice, the gallbladder was cannulated with PE-10 tubing, and both the portal vein and inferior vena cava above the liver were cannulated with a 20G catheter (Nezasa et al., 2006). After a ~15-min pre-perfusion period (FEX-free Krebs-Henseleit buffer with 5 μ M taurocholate; flow rate = 5 ml/min), single-pass perfusion of the liver was continued for 60 min with buffer containing 0.5 μ M FEX and 10 μ M GW918 or vehicle. Perfusate outflow and bile were collected *in toto* every 10 min. Rat livers were perfused in a similar, single-pass manner with Krebs-Henseleit buffer except that the common bile duct was cannulated with PE-10 tubing and the perfusate flow rate was 30 ml/min.

Analytical Methods

Livers were homogenized in three volumes (v:w) of 0.1 M phosphate buffer (pH 7.4). Homogenates were dialyzed to equilibrium (4.5 hr) against buffer using Spectra-Por2 membranes (Spectrum Laboratories, Inc., Rancho Dominguez, CA) in a 96-well plate dialysis apparatus (HTDialysis, Gales Ferry, CT) according to the manufacturer's instructions. Unbound fractions were corrected for dilution (Kalvass and Maurer, 2002).

Bile, outflow perfusate, liver homogenate and dialysate samples were analyzed by liquid chromatography with detection by tandem mass spectrometry (Applied Biosystems API 4000 triple quadrupole with TurboIonSpray interface, MDS Sciex, Concord, ON, Canada). FEX and citirazine (internal standard) were eluted from an Aquasil column (C18, 5 μ m, 50 \times 2.1 mm, Thermo Electron, Waltham, MA) using a methanol gradient. FEX and citirazine were detected

in positive ion mode using multiple reaction monitoring: FEX: 502.3 → 466.4 m/z, citirazine: 389.0 → 201.0 m/z.

Data Analysis

Based on pilot studies in which outflow FEX concentrations reached a plateau after 50 min of infusion, steady-state FEX concentrations were defined as the average concentration during the 50- to 60-min interval. The hepatic extraction ratio was calculated as the ratio of the difference between steady-state inflow and outflow FEX concentrations and the steady-state inflow concentration. Unbound intrinsic biliary clearance $CL_{bile, int}$ was calculated as the ratio of the FEX biliary excretion rate and the unbound liver concentration ($C_{liver, unbound}$). All data are reported as mean \pm SD ($n = 3-4$, per group). Statistical significance was assessed by one-way analysis of variance for rat data and two-way analysis of variance with Tukey's post hoc test for mouse data. In cases where the normality test failed, data were log-transformed prior to statistical analysis. In all cases, $p < 0.05$ was considered to be statistically significant.

Results and Discussion

The biliary excretion rate of FEX was decreased 85% in Mrp2KO mice compared to wild-type B6 mouse livers; GW918 decreased FEX biliary excretion rate ~45% in B6 and ~40% in Mrp2KO mouse livers compared to vehicle controls (Figure 1A; Table 1). FEX $CL_{bile, int}$ was significantly decreased in Mrp2KO compared to B6 mouse livers, but was not significantly affected by the co-infusion of 10 μ M GW918 in either B6 or Mrp2KO mouse livers (Table 1).

The contribution of individual canalicular transport proteins to FEX $CL_{bile, int}$ may be estimated as the difference between FEX $CL_{bile, int}$ in wild-type and transport-deficient mouse livers, assuming that other $CL_{bile, int}$ processes in the transport-deficient livers, obtained via genetic knockout or chemical inhibition, are not altered by the loss of functional proteins. Using this approach, the contribution of Mrp2 to FEX $CL_{bile, int}$ was estimated to be 1.74 ml/hr/g liver (the difference between 3.01 and 1.27), and the combined contribution of P-gp and Bcrp was estimated to be ~0.3 ml/hr/g liver (the difference between 3.01 and 2.70 for B6, and 1.27 and 1.03 for Mrp2KO livers) (Table 1). These data clearly demonstrate that Mrp2 is the major transport protein mediating FEX biliary excretion in mice, that P-gp and Bcrp play a minor role, and that at least one other unidentified transport mechanism exists, which accounts for ~one-third of FEX $CL_{bile, int}$. Although previous studies indicated that P-gp transports FEX in vitro, and that P-gp was the major efflux mechanism for FEX in the mouse intestine and brain (Cvetkovic et al., 1999), this is not the case in mouse liver. As exemplified with FEX, a positive result in an in vitro P-gp transport assay (Cvetkovic et al., 1999) does not imply necessarily that P-gp is the predominant transport pathway for the molecule in all organs that express P-gp. Tissue-/organ-specific differences in the predominant transport protein for a drug, as reported previously for paclitaxel (Jurjen et al., 2006), may be explained by transport multiplicity, including the affinity of a molecule for multiple transport proteins and the differential expression of these transport proteins in various tissues.

In contrast to mice, the biliary excretion rate and $CL_{bile, int}$ of FEX were comparable between Mrp2-deficient (TR^{-}) and wild-type Wistar rat livers (Figure 2A; Table 2), consistent with the findings of Tahara et al. (2005). One interpretation of these findings is that FEX has affinity for mouse Mrp2, but not rat Mrp2. Alternatively, other transport mechanisms in rats may exhibit greater FEX clearance due to increased affinity and/or capacity than Mrp2. The dominant activity of Mrp2 in rats, relative to other preclinical species, has been attributed to high Mrp2 expression (Ninomiya et al., 2005). The biliary excretion of glucuronide and sulfate metabolites is mediated predominantly by Mrp2 in rats, whereas in mice, Bcrp appears to play a more important role (Zamek-Gliszczynski et al., 2006a, 2006c). Clearly, species differences in hepatic canalicular transport are more complex than simple differences in Mrp2 expression

levels. This study demonstrated that impaired biliary excretion of a drug in one transporter-deficient animal model does not necessarily translate to impaired biliary excretion in another species deficient in that same transport protein. Overall, tissue-/organ-specific differences within species and inter-species differences in drug transport complicate the in vitro-in vivo correlation and allometric scaling for drugs with predominantly transporter-mediated disposition.

Mean steady-state FEX concentrations in outflow perfusate ($C_{ss, out}$) were significantly higher, consistent with decreased hepatic extraction ratios, in livers from Mrp2KO relative to B6 mice (Figure 1B; Table 1). Despite the absence of the protein primarily responsible for FEX biliary excretion, FEX liver concentrations and the liver-to-perfusate partition ratio of FEX ($C_{liver, unbound}/C_{ss, out}$) decreased significantly in Mrp2KO mice (Table 1). This apparent discrepancy reflects the enhanced function of basolateral efflux transport protein(s) that are responsible for FEX hepatic disposition in Mrp2KO mice. For example, basolateral Mrp3 and 4, which have overlapping substrate specificity with Mrp2, are up-regulated in Mrp2KO mice (Chu et al., 2006; Vlaming et al., 2006; Nezasa et al., 2006). Similarly, FEX liver concentrations were significantly lower in TR⁻ compared to Wistar rats following perfusion (Table 2). The increase in perfusate concentrations during early time points in the livers from TR⁻ relative to Wistar rats (Figure 2A) may be due to enhanced basolateral efflux of Mrp2 substrates by upregulated Mrp3 when Mrp2 is deficient.

GW918 is a more potent inhibitor of P-gp than Bcrp by an order of magnitude, thus concentrations known to inhibit Bcrp in mouse liver perfusions would also have inhibited P-gp transport (de Bruin et al., 1999). The current experiments with GW918 were designed to inhibit both P-gp and Bcrp based on mouse liver perfusion studies in which 10 μ M GW918 extensively impaired Bcrp-mediated biliary excretion of hepatically-generated 4-methylumbelliferyl sulfate (Zamek-Gliszczynski, 2006a). In the presence of 10 μ M GW918, FEX C_{liver} , $C_{liver, unbound}$ and partitioning between perfusate and liver were significantly decreased in B6 mice (table 1). FEX C_{liver} , $C_{liver, unbound}$ and partitioning between perfusate and liver tended to be lower in the presence of GW918 in Mrp2KO mice, although the differences were not statistically significant. A similar trend was observed for the extraction ratios in both B6 and Mrp2KO mice (Table 1). High concentration of GW918 (25 μ M) has been reported to inhibit Oatp2-mediated hepatic uptake of digoxin (Lam and Benet, 2004). Although GW918 did not significantly alter the $CL_{bile, int}$ of FEX in mouse livers (Table 1), consistent with normal biliary excretion of FEX in P-gp- and Bcrp-knockout mice (Tahara et al., 2005), the potential inhibition of Oatp-mediated hepatic uptake of FEX by GW918 cannot be ruled out.

In summary, this study demonstrated that FEX biliary excretion is mediated predominantly by Mrp2 in mice; the residual FEX biliary excretion in mice is due to unidentified transport mechanisms and, to a lesser extent, due to Bcrp and P-gp. In rats, the compensatory transport mechanisms responsible for FEX biliary excretion in the absence of Mrp2 remain to be identified. Alterations in liver sinusoidal transport proteins can have significant impact on the FEX biliary excretion as well. These findings highlight the complexities of in vivo drug transport processes, which are influenced by differences in transporter multiplicity and differential transport protein expression.

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List of Nonstandard Abbreviations

Bcrp	breast cancer resistance protein
B6	wild-type C57BL/6 mouse
CL_{bile}	_{int} , unbound intrinsic biliary clearance
C_{liver}	total liver concentration
C_{liver}	_{unbound} , unbound liver concentration
C_{ss}	_{out} , outflow perfusate concentration
FEX	fexofenadine
GW918	GF120918
Mrp	multidrug resistance-associated protein
Mrp2KO	Mrp2-knockout
Oatp	organic anion transporting polypeptide
P-gp	p-glycoprotein
TR⁻	Mrp2-deficient Wistar rat

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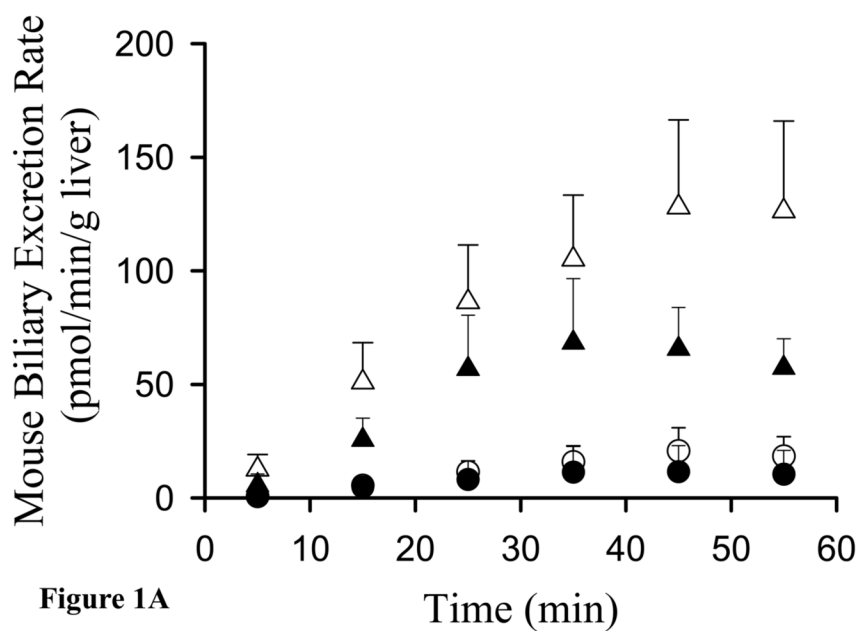


Figure 1A

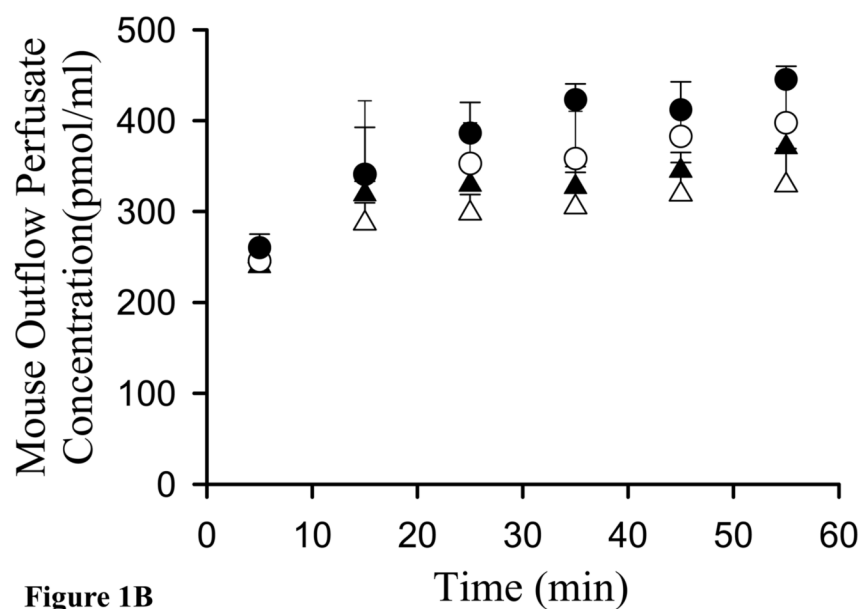


Figure 1B

Figure 1. Fexofenadine biliary excretion rates (A) and outflow perfusate concentrations (B) in perfused livers from wild-type (B6) and Mrp2-knockout (Mrp2KO) mice. Mean \pm SD, $n = 3-4$ per group. Δ , B6; \blacktriangle , B6+GW918; \circ , Mrp2KO; \bullet , Mrp2KO+GW918

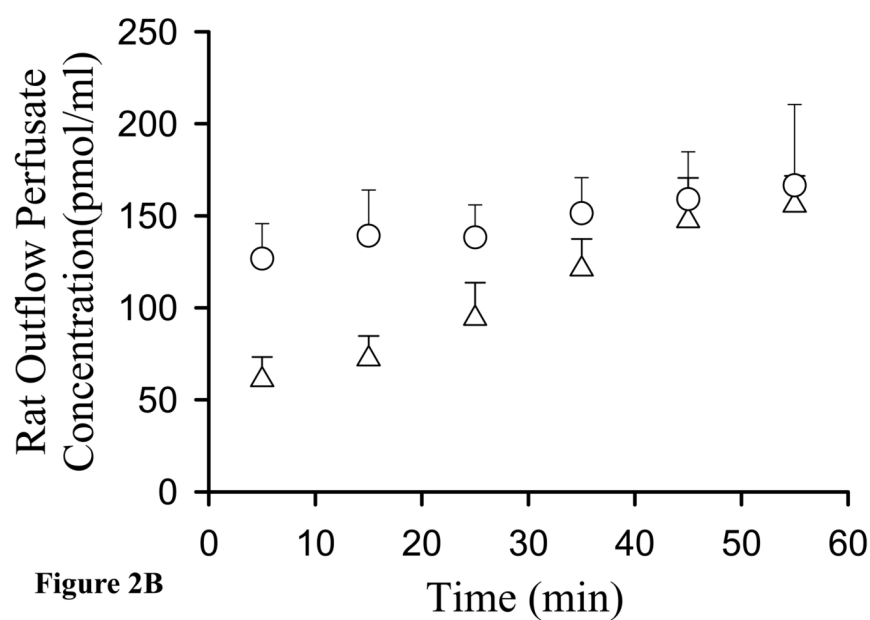
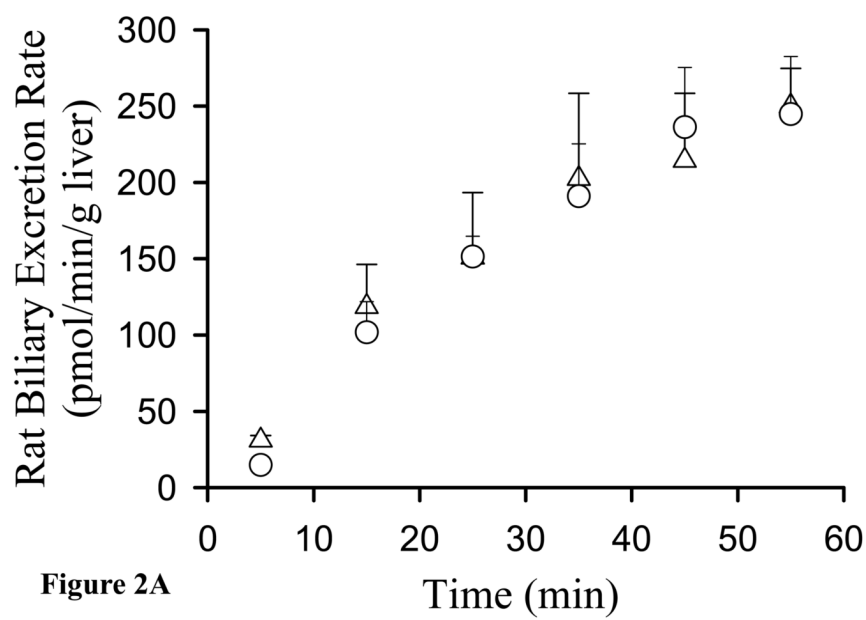


Figure 2. Fexofenadine biliary excretion rates (A) and outflow perfusate concentrations (B) in perfused livers from Wistar and Mrp2-deficient (TR⁻) rats. Mean \pm SD, n = 3–4 per group. Δ , Wistar; \circ , TR⁻.

Table 1Pharmacokinetic parameters of FEX in single-pass perfused mouse livers. Mean \pm SD, n = 3–4 per group.

Pharmacokinetic Parameters	B6	B6+GW918	Mrp2KO	Mrp2KO+GW918
$C_{ss, out}$ (pmol/ml)	329 \pm 40	371 \pm 25	398 \pm 40 [*]	445 \pm 15 [*]
C_{liver} (pmol/ml)	16261 \pm 1468	10319 \pm 2590 [§]	5913 \pm 259 [*]	4426 \pm 641 [*]
$C_{liver, unbound}$ (pmol/ml)	2428 \pm 219	1541 \pm 387 [§]	883 \pm 337 [*]	661 \pm 96 [*]
$C_{liver, unbound}/C_{ss, out}$	7.49 \pm 1.4	4.14 \pm 0.69 [§]	2.27 \pm 0.97 [*]	1.49 \pm 0.21 [*]
Extraction ratio	0.34 \pm 0.08	0.26 \pm 0.05	0.21 \pm 0.08 [*]	0.11 \pm 0.03 [*]
Biliary excretion rate (pmol/min/g liver)	122 \pm 34	66.8 \pm 7.9 [§]	18.4 \pm 2.2 [*]	11.2 \pm 1.7 ^{§*}
$CL_{bile, int}$ (ml/hr/g liver)	3.01 \pm 0.65	2.70 \pm 0.64	1.27 \pm 0.31 [*]	1.03 \pm 0.18 [*]

[§] p < 0.05, vehicle vs GW918;^{*} P < 0.05, Mrp2KO vs B6 control mice

Table 2Pharmacokinetic parameters of FEX in single-pass perfused rat livers. Mean \pm SD, n = 3–4 per group,

Pharmacokinetic Parameters	Wistar	TR ⁻
C _{ss, out} (pmol/ml)	156 \pm 16	167 \pm 44
C _{liver} (pmol/ml)	15450 \pm 490	10117 \pm 2043 [*]
C _{liver, unbound} (pmol/ml)	2310 \pm 73	1511 \pm 305 [*]
C _{liver, unbound} /C _{ss, out}	14.8 \pm 1.6	9.05 \pm 3.0 [*]
Extraction ratio	0.68 \pm 0.03	0.63 \pm 0.1
Biliary excretion rate (pmol/min/g liver)	250 \pm 25	245 \pm 38
CL _{bile, int} (ml/hr/g liver)	6.49 \pm 0.68	9.72 \pm 2.5

*
p < 0.05, TR⁻ vs. Wistar